Endothelial Function and Dysfunction in the Cardiovascular System: The Long Non-Coding Road

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ABSTRACT

Present throughout the vasculature, endothelial cells are essential for blood vessel function and play a central role in the pathogenesis of diverse cardiovascular diseases. Understanding the intricate molecular determinants governing endothelial function and dysfunction is essential to develop novel clinical breakthroughs and improve knowledge. An increasing body of evidence demonstrates that long non-coding RNAS (lncRNAs) are active regulators of the endothelial transcriptome and function, providing emerging insights into core questions surrounding endothelial cell contributions to pathology, and perhaps the emergence of novel therapeutic opportunities. In the present review, we discuss this class of non-coding transcripts and their role in endothelial biology during cardiovascular development, homeostasis, and disease, highlighting challenges during discovery and characterisation and how these

have been overcome to date. We further discuss the translational therapeutic implications and the challenges within the field, highlighting lncRNA that support endothelial phenotypes prevalent in cardiovascular disease.

1. INTRODUCTION

Lining the totality of the vascular system, the endothelium is a continuous barrier, the surface of which directly interacts with nearly every system in the body to regulate vascular development, homeostasis and pathogenesis. The single layer of endothelial cells (EC) that constitutes this barrier is in itself uniquely versatile, showing remarkable physiological and morphological heterogeneity across the vasculature[1]. These differences reflect the variety of functions they perform and the shifts in the priority of these roles across different anatomical locations in the cardiovascular system. For instance, the angiogenic capacity of endothelial cells plays a vital role during embryological growth, tissue development, and wound healing in damaged tissues[2]. Maintenance of vessel function, and therefore cardiovascular homeostasis, is highly dependent on the ability of the endothelium to react to external stimuli, mediating not only vasodilation and constriction but also thrombogenic, immune and inflammatory responses[3,4]. Dysregulated, these mechanisms can lead to, among others, the unrestrained vessel formation often seen in cancerous tumours[5] and the pathological remodelling of mature vessels associated with the development of pervasive conditions such as hypertension[6] and atherosclerosis[7]. Unsurprisingly, endothelial dysfunction is often an early pathophysiological feature in most forms of cardiovascular disease (CVD) and an independent predictor of future cardiovascular outcomes[8,9]. Understanding the finer molecular determinants governing endothelial function and dysfunction is therefore essential when addressing vascular disease.

Apparent during both health and disease, the unique versatility of endothelial cells indicates complex control of the underlying transcriptional programmes and this is becoming more apparent with advances in our understanding of the human transcriptome. This is particularly true with the emergence of non-coding RNAs (ncRNA) as regulators of gene expression both at the transcriptional and post-transcriptional level. With only an estimated 1-2% of the human genome encoding for proteins[10], there have been increased efforts in recent years to uncover novel regulatory mechanisms within the uncharacterised portion of the transcriptome. While the extent of their function is still debated[11] ncRNA transcripts are believed to comprise approximately 70 to 90% of our genome and encompass thousands of operationally significant RNAs associated with all manner of biological processes[12,13]. Whilst examples of small non-coding RNA-mediated regulation are now well established, the concept of widespread control of cell function by long non-coding RNA (lncRNA) has only been advocated within the last decade[14]. This class of genes, initially thought to only contain non-functional transcriptional by-

products, has sparked great interest as some have been found to be critical in development and dysregulated in disease[15].

Widespread transcription of lncRNA across the genome became more apparent with the increased use of RNAseq technologies revealing previously undetected transcriptional activity. Defined simply as non-coding transcripts longer than 200bp in length, these genes are generally less abundant and far less conserved compared to protein coding genes. Indeed, in a survey of 17 species, over 70% of all lncRNA in each species originated within the last 50 million years, indicating a high rate of evolutionary turnover within the class[16]. Recent estimates describe approximately 27,000 lncRNA transcripts produced in human, and ~1,000 lncRNA are conserved in other mammals[16,17].

LncRNAs collectively evade exact definition, with a variety of intersecting subclasses described based on traits such as their genomic proximity relative to surrounding genes (antisense, intergenic, bidirectional, intron-contained), the chromatin signatures of their transcriptional start sites (promoter, enhancer) and their mode of regulation (transcriptional vs post-transcriptional)[18]. Relatively few have been characterised compared to the tens of thousands of genes annotated, but those that have demonstrate a wide range of mechanisms with epigenetic, transcriptional and post-transcriptional effects, able to activate or suppress gene expression and translation[19,20] (Fig. 1). Such diversity leads to challenges during the study of lncRNA, but their prevalence in the transcriptome provides an attractive opportunity for discovery of new mechanisms that control cell behaviour and the possibility of developing novel clinical targets.

Discovery of new clinical targets is a current priority for pathologies where endothelial dysfunction plays a significant role and treatment options are limited. Aside from providing an opportunity to find novel regulatory mechanisms to manipulate therapeutically, lncRNAs often show restricted expression patterns that could be clinically advantageous[21]. Using *in situ* hybridisation data, leading studies by Mercer and colleagues found lncRNA to have expression patterns associated with particular anatomical regions, cell types and subcellular compartments[22]. Tissue-specificity is high for lncRNAs, often surpassing that of protein-coding genes[23]. Additionally, they often have stage-specific expression patterns during development and disease[24,25]. Whether such tendencies for localised expression could potentially explain some degree of endothelial heterogeneity through regulation of transcriptional programmes in certain vessel types or tissues is currently an open question.

Herein, we will discuss the role of lncRNA in endothelial biology during cardiovascular development, homeostasis and disease and how this is yet to be fully defined. We will highlight challenges during lncRNA discovery and characterisation and how these have been overcome so far, using as examples those lncRNA that support endothelial phenotypes prevalent in cardiovascular disease (CVD).

2. LNCRNA DISCOVERY IN ENDOTHELIAL CELLS: CURRENT STATE OF THE ART

As interest in lncRNA function grows, a wealth of established *in vitro* and *in vivo* models are being used as a basis to study their expression patterns. Such models are yet to be thoroughly examined from a lncRNA perspective and a full representation of endothelial lncRNA expression throughout the cardiovascular system is yet to be realised, although clearly a wealth of data will emerge in the next months and years. To date, a number of studies on EC function have identified lncRNAs with clear functional impact on the endothelial cell (Table 1). For instance, hypoxic conditions are commonly observed in myocardial infarction, peripheral ischemia and stroke, these often trigger a variety of distinct endothelial responses to prevent further tissue damage and restore blood supply. Several hundred genes with hypoxia-sensitive expression have already been identified and later validated *in vitro* using human umbilical vein endothelial cells (HUVECs), including *MALAT1*, *H19*, *MIR503HG* and *LINC00323*. Many of these have been shown to regulate hypoxia-induced EC functions such as proliferation, migration and angiogenesis after further *in vitro* and *in vivo* characterisation[26–28]. Additionally, lncRNAs previously characterised in other cell types, such as *MEG3*, *MIAT* and *RNCR3*, have been tied to EC-mediated angiogenesis via hyperglycaemic induction in retinal EC and are now associated with microvascular visual impairment[29–31] (Fig. 2).

LncRNA involved in EC inflammatory responses have also been explored. *In vitro* lipopolysaccharide-stimulation of ECs can cause apoptosis, endothelial dysfunction and propagation of sepsis leading to elevated CVD risk. Hundreds of so far uncharacterised lncRNA have been found to be differentially expressed during the process, several with notably high fold changes[32]. A key feature of an innate EC response are the chemotactic intermediaries produced from the CXCL locus involved in neutrophil recruitment. These were recently found to be primed for activation in TNFα-stimulated HUVECs by *UMLILO*, a proximal enhancer-transcribed RNA[33]. Such innate-responsive lncRNAs are likely to directly impact on endothelial function through pro-apoptotic or pro-migratory effects on ECs.

Models of endothelial development from human embryonic stem cells (hESCs) have also been used to identify lncRNA expressed at specific stages in cell fate determination. Such differentiation models have identified genes such as *SENCR*[34] and *PUNISHER (AGAP2-AS1)*[25], which are both upregulated during endothelial commitment. While differentiation models allow for identification of genes involved in determining cell fate, many may also be important in maintaining endothelial homeostasis. *SENCR* expression, for example, was shown to be altered in the vascular tissue of patients with critical limb ischemia and premature coronary artery disease[34]. Later studies have also found perturbations in mature EC membrane integrity induced upon *SENCR* knockdown[35].

However, publications often do not acknowledge endothelial phenotype heterogeneity and several EC-dependent mechanisms have yet to receive similar attention as those supporting angiogenesis and homeostasis. For example, the well-established endothelial-mesenchymal transition (EndMT) has so far only been associated with a single lncRNA, *GATA6-AS* [36,37], with no published data of transcriptome-wide shifts in lncRNA expression during this transition.

A bias towards HUVEC-based models is also apparent with only a single lncRNA-screening study including an EC subpopulation from outside the umbilical vein published so far[38]. This is of particular importance as studies highlight distinct LncRNA functions across vessel types; knockdown of the lncRNA *MIR503HG* for example, was shown to have anti-proliferative and migratory effects on venous EC but not on arterial EC[27]. Further, given the wide variety of tissue microenvironments to which EC are exposed is pivotal to guiding their fundamental function[39], it is important to acknowledge the extracellular cues that may dictate some of these differences in phenotype. *In vitro*, the use of 3D multicellular co-culture models allow for a physiologically relevant cellular arrangement and the exchange of intracellular components such as protein, RNA and DNA[40]. The release of extracellular vesicles (EVs) by neighbouring cells is one such cellular exchange system shown to be a key regulator of endothelial function and dysfunction[41]. Some emerging evidence now indicates that lncRNAs can be selective packaged into EVs to induce a variety of phenotypic changes under both physiological and pathological conditions[42]. For instance, exosomes released from tumour cells containing high quantities of the lncRNA *H19* promote EC network formation, stimulating VEGF and VEGFR1 production[43]. Exosomal release of the atherosclerosis-associated lncRNA *GAS5* by monocytes was also found to regulate apoptosis of vascular endothelial cells[44]. Conversely, communication between ECs and vascular smooth muscle cell (VSMC) has been shown to be facilitated by EVs carrying the lncRNA *RNCR3*, and that these EC-derived exosomes can induce VSMC proliferation and migration[45].

Ultimately, research addressing EC heterogeneity and the physiological relevant cues that surround these subpopulations has the potential to reveal an unexplored and therapeutically attractive subset of lncRNA. As novel sequencing data and protocols become available to tackle these cellular mechanisms and typically hard-to-culture cell populations, annotation of EC functional diversity in the literature is likely to improve over the next years. It is, therefore, important to acknowledge transcriptome-wide discovery as a prerequisite for understanding EC function and to consider the available emerging tools and techniques.

3. STRATEGIES FOR DETECTION AND SELECTION OF FUNCTIONAL ENDOTHELIAL LNCRNA

In the last decade the advent of relatively affordable sequencing technology has created a boom in transcriptomic techniques, resulting in sophisticated RNAseq-based methods becoming available to scientists and routinely utilised as a discovery tool[46]. In tandem, the methodology to quantify gene expression has been refined[47], providing an ever-more accurate account of transcriptional fluctuations within models, including for endothelial pathology and differentiation. This work has helped to expand and validate lncRNA annotations where sequencing depth is effective, as well as increase our understanding of their origins, structure and expression. However, the specific spatiotemporal expression of lncRNAs poses a challenge, and the annotation of the human transcriptome is still

incomplete and likely missing transcripts, including lncRNAs, that are well-expressed in EC types and behaviours that have not been profiled yet. Detecting novel endothelial lncRNA is therefore a high priority. This is in addition to evaluation of the thousands of already annotated lncRNA for endothelial association. Here we provide an overview of the process of identifying lncRNA coupled to the subsequent selection of high priority candidates that might be functionally relevant in endothelial cells (Fig. 3).

Microarrays, though now superseded in sensitivity and accuracy by RNAseq, have provided the basis of several important screens for endothelial lncRNAs[32,48,49]. However, the necessity to decide on which annotation set the probe population will be based on eliminates the chance to find novel transcripts, even if using arrays specifically designed for lncRNAs. This is coupled with increased background noise from non-specific probe binding, which limits sensitivity particularly in regard to those lncRNA that are expressed at low levels. However, arrays can provide a relatively cheap alternative to deeper RNAseq methods in studies with high sample number, such as those screening multiple vascular cell types[38], or where many replicates are required to obtain a signal of co-expression with genes of interest[50].

The gold standard technique for lncRNA identification is RNAseq. It is sensitive, increasingly cost-effective and presents a largely unbiased, whole-transcriptome view of shifts in gene expression. RNAseq of ECs has been used to identify lncRNAs responding to hypoxia[26–28,37], pathological shear stress[51] and those expressed in developmental models[25]. It has also allowed profiling of transcriptional activity at loci containing notable endothelial-expressed genes such as eNOS[51] or SR1P[52]. However, only one of these studies capitalises on the ability to detect expression of completely novel transcripts outside of current annotation sets and this led to identification of *PUNISHER*[25]. Other such novel lncRNA are rare in the literature at present though the previously unannotated *SENCR*, has been characterised with an endothelial function[34] despite being initially identified in SMCs[53]. Interestingly, both these functional lncRNA are expressed in unstimulated HUVECs, which have a high amount of sequencing data readily available, yet both were found without the use of publicly available annotation sets. This demonstrates that even well-studied cell types can yield previously unseen transcripts with high phenotypic impact.

A key caveat for use of RNAseq is that sufficient read depth must be used to be able to reliably detect and annotate lowly expressed lncRNAs. This problem is even more acute when considering use of single cell RNA-seq to identify lncRNA expression on a cell-specific level. An important recent study showed how lncRNA expressed at a low level in a given tissue can actually be highly abundant within certain populations of the constituent cells[54]. This study used 200 million reads per sample – a prohibitively expensive amount of sequencing until recently, but increasingly affordable with the introduction of NovaSeq machines[55]. The use of single cell RNA-seq to examine lncRNAs directing endothelial heterogeneity, transitions or development remains an area of high interest and opportunity.

The use of RNAseq data for prediction of new lncRNAs is typically based on algorithmic reconstruction of transcript models from short RNAseq reads (these algorithms are usually 'seeded' with existing annotations), followed by filtering to determine which transcripts are noncoding with high confidence[56]. Several computational pipelines address these issues[57–60]. Typical filtering steps include the use of several algorithms to evaluate coding potential and combination of their scores, exclusion of short or very lowly expressed transcripts, and exclusion of transcripts found in close proximity to annotated genes, as these often correspond to unannotated extensions of UTRs.

For example, transcripts containing long ORFs (i.e. possessing long stretches of uninterrupted sequence between in-frame start and stop codons) with characteristic codon frequencies and/or with high homology to existing proteins can be identified with the widely-used coding prediction calculator (CPC) scoring method[61]. Others exclude candidates with ORFs predicted to produce proteins with structural homology to known Pfam protein domains (HMMR)[62], the detection of sequence composition characteristic of coding sequences[58,63], or candidates containing ORFs with codons that are maintained (with the same or similar codons and without frameshifting mutations) over large evolutionary distances (RNAcode and PhyloCSF)[57]. The codon-conservation tools are particularly powerful for detecting short conserved ORFs, but also can have some false positives in regions of extremely high conservation and limited variation between species. Concepts relevant to all these tools are reviewed elsewhere[64], there remains an opportunity for such approaches to expand the endothelial lncRNA repertoire and aid our understanding of endothelial cell function.

Analysis of high-throughput data typically identifies tens to hundreds of potentially relevant lncRNAs and selection of candidates for functional follow-up can be challenging. There is no silver bullet available for homing in on a prospective lncRNA on which to focus wet-lab resources. There are, however, general trends emerging from the accumulated knowledge of lncRNA which provide good starting points[11]. LncRNAs with substantial transcript abundance and differential expression are most likely to be associated with the phenotype in question. Expression levels also correlate with evolutionary conservation[65], indicating that more abundant genes are more likely to be maintained for functional importance across species. On the other hand, lncRNAs that have *cis*-acting scaffold functions do not necessarily need to be abundant for carrying out their function(s), as exemplified by the involvement of *UMLILO* in regulation of the CXCL locus in HUVECs[33].

Sequence conservation also offers a route to prioritize functional lncRNA[66]. LncRNAs are in many cases poorly conserved, but ~1,000 lncRNA genes are conserved among mammals, and ~100 between human and fish[16]. The regions conserved in sequence in these lncRNAs are typically short, in many cases restricted in just a single exon with some bias towards the 5' of the RNA. These short patches of conservation may correspond to functional domains with autonomous functions such as a binding sites for other RNAs or proteins. BLAST or whole genome alignments can be used to find regions of significant sequence similarity between potentially orthologous transcripts. Some lncRNA are

conserved only through synteny, where only the relative position and the orientation of the transcribed locus of transcription is constrained whilst sequence diverges to a point where significant similarity is no longer detectable[66]. Such 'positional' orthologs are harder to call with confidence, as multiple transcripts can be present in the same locus in distantly related species. Discovery of all such relationships can be automated using a relatively easily implemented tool, *slncky*, which can be used to annotate lncRNA in sparsely-annotated cardiovascular animal models and then predict their orthologous relationships to human transcripts[59].

The presence of deeply conserved orthologs can imply a significant contribution of a lncRNA to endothelial homeostasis or development. Such genes are therefore strong candidates for targeting in functional studies. Conversely, human- or mouse-specific transcripts may very well also be functional, and many of these could also potentially add to our understanding of differences between pre-clinical animal models and an eventual clinical context. Prioritization of such transcripts is typically based on their expression levels and/or proximity to functionally-relevant genes.

The selection process will become better informed as the list of functionally characterised lncRNA grows; a greater number of validated genes will allow for clearer guidelines for effective selection to emerge. Until then potential key drivers of endothelial heterogeneity or pathology could be waiting to be unearthed.

4. APPROACHES FOR CHARACTERISING THE MOLECULAR INTERACTIONS OF LNCRNAS

Being highly diverse, lncRNAs carry out their functions through a wide range of mechanisms. At the transcriptional level, they have been shown to bind and guide chromatin-modifying complexes to specific DNA sequences, either activating or repressing their target gene expression[67,68]. Additionally, they can also act as decoys for DNA-binding proteins and prevent their association to a target gene[69]. At the post-transcriptional level, a growing number of studies have implicated lncRNAs at various stages of control, regulating mRNA stability[70], enhancing mRNA translation[71] and even acting as miRNA sponges[72]. Recent publications also point towards the existence of widespread cross-regulatory interactions between noncoding RNAs classes, adding a further functional role for lncRNAs[73]. Some of these regulatory networks are represented in large-scale databases, such as StarBase v2.0, a repository of thousands of experimentally validated RNA–RNA and protein-RNA interactions that offer supporting information for mechanistic studies[74]. Nevertheless, the use of appropriate RNA-focused tools to elucidate such complex interactions is essential given the variety of different interactions across not only between different tissues and cell types, but also within same-cell subpopulations.

CELLULAR LOCALISATION

In line with their diverse functions, lncRNAs show a variety of subcellular distributions: accumulating predominantly either in the cytoplasm, nucleus or distributed between both compartments[56]. Our understanding of how the localization is encoded in lncRNA sequences is still relatively rudimentary, but several recent studies have identified specific sequence elements[75,76] other features that are associated with enrichment in the nucleus in lncRNAs and mRNAs[77]. Understanding the localisation of a transcript may thus give an initial insight into putative functions and serve to guide future experiments.

Isolation of nuclear and cytoplasmic extracts has proven to be a simple, yet effective strategy. For example, different subpopulations of EC express high levels of the nuclear enriched lncRNAs *TUG1*, *MEG3*, and *MALAT1*[26], whereas *SENCR* transcripts show both cytoplasmic and nuclear accumulation[53]. Unsurprisingly, all of these have now been found to regulate different aspects of EC function[26,35,78,79]. Such fractionation methods, however, give a crude resolution quantification of subcellular localization that should be accompanied by more detailed localization through the use of single-molecular FISH methodologies[80].

RNA:PROTEIN INTERACTION

The past decade has seen a growing appreciation for the role of RNA:Protein interactions in regulating gene expression. These interactions can be studied either through protein-focused and RNA-focused perspectives. Well-established immunoprecipitation assays include RNA Immunoprecipitation (RIP), which purifies full RNA molecules associated with the precipitated protein, and Cross-Linking Immunoprecipitation (CLIP), which is typically used to identify higher-resolution footprints of RNA binding proteins[81]. Conversely, RNA-centric approaches such as RNA chromatography will require the *in vitro* generation of a labelled RNA of interest to identify binding partners[82], other hybridization-based methods such as RNA antisense purification (RAP)[83,84] will instead use labeled antisense oligos to purify the endogenous RNA instead. While these techniques have now expanded and become quite varied in their methodology, in general they allow for the purification a specific RNA complex to identify directly interacting proteins using quantitative mass spectrometry or western blot (Fig. 4). Nonetheless, while there has been progress in understanding the interactomes of individual lncRNAs, such as *HOTAIR*[85], *MALAT-1*[86] and *Xist*[87], the majority are still not well understood, particularly within an endothelial-specific context.

In endothelial cells, RNA chromatography-based approaches have been extensively used. For example, in order to uncover the interacting partners of the nuclear enriched endothelial lncRNA *MANTIS* Leisegang and colleagues exposed 3'end biotinylated transcripts to EC nuclear extracts, allowing for RNA:Protein complexes to be formed which were then be captured and isolated[49]. This study identified an interaction of *MANTIS* with Brahma-like gene 1 (BRG1), a subunit for the chromatin-remodelling

complex SWI/SNF. This interaction was shown to regulate SMAD6, SOX18, and COUP-TFII expression, leading to the increased angiogenic function associated with the lncRNA[49]. A Similar *in vitro* strategy was used to identify a direct interaction between SENCR and the cytoskeletal-associated protein 4 (CKAP4), via a noncanonical RNA-binding domain. This CKAP4:*SENCR* association was found to indirectly stabilize CDH5 at the adherens junction and thus maintain normal EC membrane homeostasis[35]. Nonetheless, despite its success, this system relies on the *in vitro* transcription of the lncRNA rather than targeting endogenously expressed transcripts present in the cell, which is prone to the formation of non-physiological interactions.

More recently, biotin-labelled RNA antisense probes were used to investigate endogenous *GATA6-AS*:protein complexes in HUVEC. The study demonstrated that this lncRNA exerted its effects on endothelial cell function by binding the nuclear enzyme LOXL2 and impairing its deamination activity on H3K4me3, which is accompanied by transcriptional silencing of a variety of endothelial genes[37].

RNA:RNA INTERACTION

Much like miRNAs, snoRNAs, and tRNAs, lncRNAs can directly interact with other RNA transcripts though direct base pairing. Several strategies to uncover these RNA:RNA interactions have started to emerge, many of which have successfully been used to understand the mechanistic action of ribosomal RNA and small nuclear RNAs.

Similarly to RNA:protein detection approaches, modified versions of RAP can be used to detect lncRNA:RNA interactions[88]. These techniques rely on RNA capture using one or several antisense oligonucleotides followed by RNA sequencing. Various cross-linking methods (i.e. 4'-aminomethyltrioxalen, formaldehyde, disuccinimidyl glutarate or ultra-violet irradiation) can be used depending on the interactions of interest, be it only direct RNA:RNA interaction or indirectly via a protein intermediates[88]. Techniques such as CLASH (Cross-linking, ligation and sequencing of hybrids), for example, take advantage of UV cross-linking to induce the formation of covalent RNA bonds to identify RNA–RNA interactions occurring in close physical proximity. This method has led to the experimental identification of more than 18000 miRNA:RNA interactions[89].

Highly expressed lncRNAs can potentially act as competing endogenous RNAs (ceRNAs) or sponges to regulate the expression of other RNAs, be it non- or protein coding[90]. Large bioinformatic databases can assist in the discovery of such interactions. For example, based on StarBase v2.0 predictions, Shan and colleagues found that the EC-associated lncRNA *RNCR3* could regulate the expression of the atheroprotective Kruppel-like factor 2 (KLF2) by binding to miR-185-5p, a post-transcriptional regulator of KLF2[31].

RNA:DNA INTERACTION

LncRNAs are key regulators of chromatin states in a variety of biological processes, both in *cis*, via the regulation of neighbouring genes, or in *trans* to regulate distantly located genes. While lncRNA:DNA co-localisation can be validated using fluorescent probes, FISH-based strategies have limited ability to identify DNA regions bound by lncRNAs and higher resolution methods are needed. RNA-based methods for high-throughput identification of chromatin regions bound by lncRNAs have recently emerged. As with RNA:protein and RNA:RNA interactions, RNA capturing techniques can be modified to target chromatin interactions, these include chromatin isolation by RNA purification (ChIRP), capture hybridization analysis of RNA targets (CHART) and RAP-DNA.

First introduced in 2011, ChIRP is now widely used to identify associations between a lncRNA of interest and chromatin. As with some of the previous methodologies described, the samples are crosslinked and biotin labelled oligonucleotides targeting a lncRNA of interest[91]. The lncRNA:DNA complexes can then be purified using biotin-binding, magnetic streptavidin beads and then sequenced to identify any associated genomic regions[91]. In its original study, ChIRP was used to isolate DNA regions associated with the lncRNA *HOTAIR*, showing that the lncRNA preferentially associates with GA-rich DNA motifs[91]. A similar approach was recently used to fine-map genome-wide *MEG3* binding sites. Using 15 biotin-labelled antisense DNA oligonucleotides (oligos) spanning across lncRNA, the pulldown found that the genomic regions of 5,622 genes can be associated with *MEG3*, including several TGF-β pathway genes[92]. *MEG3* expression has since been consistently linked to endothelial cell migration, proliferation and angiogenesis[79,93,94].

5. LNCRNA DISCOVERY, FUNCTION AND TRANSLATION: CHALLENGES AND FUTURE DIRECTIONS

Whilst the therapeutic potential of lncRNAs is mentioned in most studies, real-world examples of tissue-specific clinical applications based on lncRNAs remain limited, with many emerging pre-clinical studies focusing on their utility as markers of disease. This is particularly true in cancer research, where *MALAT1* overexpression has been linked to tumour development and progression in a variety of tissues[95]. A recent meta-analysis of 14 independent studies comprising data from 1373 patients found a significant association between *MALAT1* expression and survival rates, independent of cancer type or country of residence[96], making it not only a powerful prognostic marker for cancer patients and an attractive target for anti-metastatic therapy. Similarly, the lncRNA *HOTAIR* has also been put forward by multiple studies as a potential biomarker for various cancers involving breast, liver, gastric, lung, and oesophagus[97].

Throughout the various aetiologies of cardiovascular disease, some lncRNAs markers, whilst not endothelial-specific, have been reported. A study comparing the expression of lncRNAs in peripheral

blood cells of healthy volunteers and myocardial infarction patients, demonstrated that the lncRNA *HIF1αAS2*, *KCNQ1OT1*, *MALAT1* were significantly upregulated in myocardial infarction patients[98]. In a recent clinical study of coronary artery disease (CAD), the lncRNA *CoroMarker* was used to successfully identify 78% of CAD patients out of 221 CAD patients and 187 control individuals[99]. Importantly, *CoroMarker* is mainly present in circulating extracellular vesicles, which are stable in plasma and easy to use as a biomarkers[99]. The lncRNA *LIPCAR* is consistently upregulated in the plasma of ischemic and non-ischemic heart failure patients and has been consistently reported to be an independent predictor of cardiovascular-related death[100,101].

Expressed in vascular endothelial cells, macrophages and coronary smooth muscle cells, higher plasma levels of the lncRNA *ANRIL* have been found to be associated with the incidence of in-stent restenosis (ISR)[102]. Considering the importance of inhibiting neointimal proliferation while promoting re-endothelialisation after vascular stenting[103], along with the lncRNAs reported regulation vascular endothelial growth factor (VEGF) expression and function in primary EC[104], *ANRIL* may ultimately be an optimal prognostic factor for ISR. Additionally, a meta-analysis of 14 genome-wide association studies highlighted *ANRIL* as a crucial locus of genetic sensitivity for coronary artery disease (CAD). Several single nucleotide polymorphisms (SNPs) in this locus, influencing *ANRIL* function and levels of expression, have since been found to be associated with increased susceptibility to CAD and diabetes[105,106]. Lastly, *ANRIL* has also been consistently found to be upregulated in plaque and plasma of atherosclerosis patients, making it a potential biomarker for atherosclerosis[107,108].

The introduction of lncRNA targeting strategies into the clinical setting comes with many challenges. Due to the pleiotropic nature of some lncRNAs, tissue-specific delivery is imperative to guarantee treatment efficiency and minimal off-target effects. While certain transcripts may be involved in particular pathologies, their desired function may be tissue-specific and even cell-type–specific. For example, while *ANRIL* expression may be a prognostic factor for ISR and atherosclerosis, systemic modulation of this lncRNA may prove to be harmful as it is one of the most commonly altered lncRNAs during cancer development and progression, including ovarian cancer, breast cancer and lymphoblastic leukemia[109]. Additionally, with studies showing an average of ≃4 different isoforms per lncRNA, the transcriptional complexity of a particular lncRNA locus must be thoroughly characterised before treatment strategies can translated into the clinical setting[110]. As such, effective therapies targeting or using lncRNAs in the clinical setting must take into account not only possible off-target effects, the route of delivery used, drug immunogenicity, treatment dosage and duration but also sub-cellular transcript location, transcript size and sequence.

Further, the lack of sequence conservation across different species makes the translation of pre-clinical animal studies extremely challenging. Take for example the lncRNAs myocardial infarction-associated transcript 1 (*MIRT1*) and 2 (*MIRT2*) which, while found to be associated with left ventricular remodelling after myocardial infarction, have no corresponding human homologs[111]. Conversely,

smooth muscle enriched lncRNA (*SMILR*) seems to be conserved only in humans, significantly limiting future preclinical studies using animal models and possible clinical translation[112]. However, promoter regions of lncRNAs, have in fact been shown to harbour substantial conservation throughout different organisms, comparable to that of protein-coding genes[113]. Further, it is possible that in some cases, despite divergent sequences, lncRNA sub-structures may be conserved between human and model organisms[114,115] and so small molecules targeting these structures might have similar effects across species.

Finally, given their high spatiotemporal expression specificity, certain lncRNA may allow to circumvent the need for endothelial-targeting tools with minimal off target effects. Take for example the Wisp2 super-enhancer–associated lncRNA (*WISPER*), whose expression and function are highly specific to cardiac fibroblasts, making it an attractive candidate for targeted antifibrotic therapies[116]. With continued implementation, novel RNA discovery and characterisation techniques have the potential to reveal previously unknown subsets of EC-specific lncRNAs.

6. CONCLUSIONS

An increasing body of evidence demonstrates that lncRNAs are active regulators of endothelial function. It is necessary to further define both the key lncRNA candidates and the molecular partners involved at a mechanistic level. Greater variety in lncRNA profiling in diverse ECs is clearly required. Increased lncRNA annotation in animal EC models is possibly an even greater challenge and it is crucial to expand in vivo evidence of causality. The continued emergence of high-throughput datasets has identified several attractive candidates, yet these are often far from fully characterised mechanistically. Meeting these challenges is only possible with continued screening efforts using cutting-edge RNAseq techniques paired with robust RNA capturing tools, this is particularly important with the emergence of single-cell RNA sequencing offering further insights into cell transcriptome heterogeneity in ECs. As more individual lncRNA are characterised, and as the class as a whole is further investigated, their general categorisation, characteristics and quirks become ever more clear. The field is now poised to answer whether lncRNA are intrinsically linked to broad questions surrounding endothelial heterogeneity and fate. For instance, the spatiotemporal expression of lncRNA could fit well with fundamental differences between endothelial cells derived from various vessel types or other vascular cell types. With the potential to contribute answers to such pertinent questions, alongside their potential as precise modulators of ECs via gene therapy, it is only a matter of time until a more fully-fledged and representative landscape of EC lncRNA emerges.

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LEGENDS

Table 1 - LncRNAs Associated with Endothelial Function in Cardiovascular Disease. List of lncRNAs reported to have endothelial regulatory functions in cardiovascular disease. LncRNAs are presented together with their type and evolutionary conservation, followed by details regarding their identification and characterisation in endothelial cells.

Figure 1 - LncRNA Classification and Function. LncRNA can be categorised based on their transcription start site, surrounding coding genes and regulatory function. Promoter-derived transcripts are more likely to be spliced, poly-adenylated, lengthy and stable as compared to enhancer-derived which, though separately categorised as eRNAs, are included in lncRNA studies if >200bp. The position of a lncRNA relative to surrounding coding genes as a categorisation method provides indication of potential lncRNA function e.g. bidirectional transcripts likely impact their partner gene. LncRNAs whose function has been characterised fall into distinct groups based on the its molecular interactions and the mechanism of action used to impact gene transcription or translation. As part of RNP component, lncRNAs can regulate the activity or localization of a particular protein, or play a structural role within a larger protein complex. LncRNAs can also control gene transcription by acting as enhancer RNAs, by modulating the activity of transcription factors, or through the recruitment of chromatin modifying complexes. In addition to acting as miRNA host genes, lncRNA can function as miRNA sponges, titrating specific miRNAs away from their mRNA targets. Post-transcriptionally, lncRNAs have been shown to modulate both mRNA translation and degradation.

Figure 2 - Known Function of Endothelial LncRNA. List of lncRNA reported to have endothelial regulatory functions with impact on cell differentiation, EndMT, angiogenesis, inflammation, EHT and vessel homeostasis. EndMT = Endothelial-to-mesenchymal transition, EHT = Endothelial-to-haematopoietic transition.

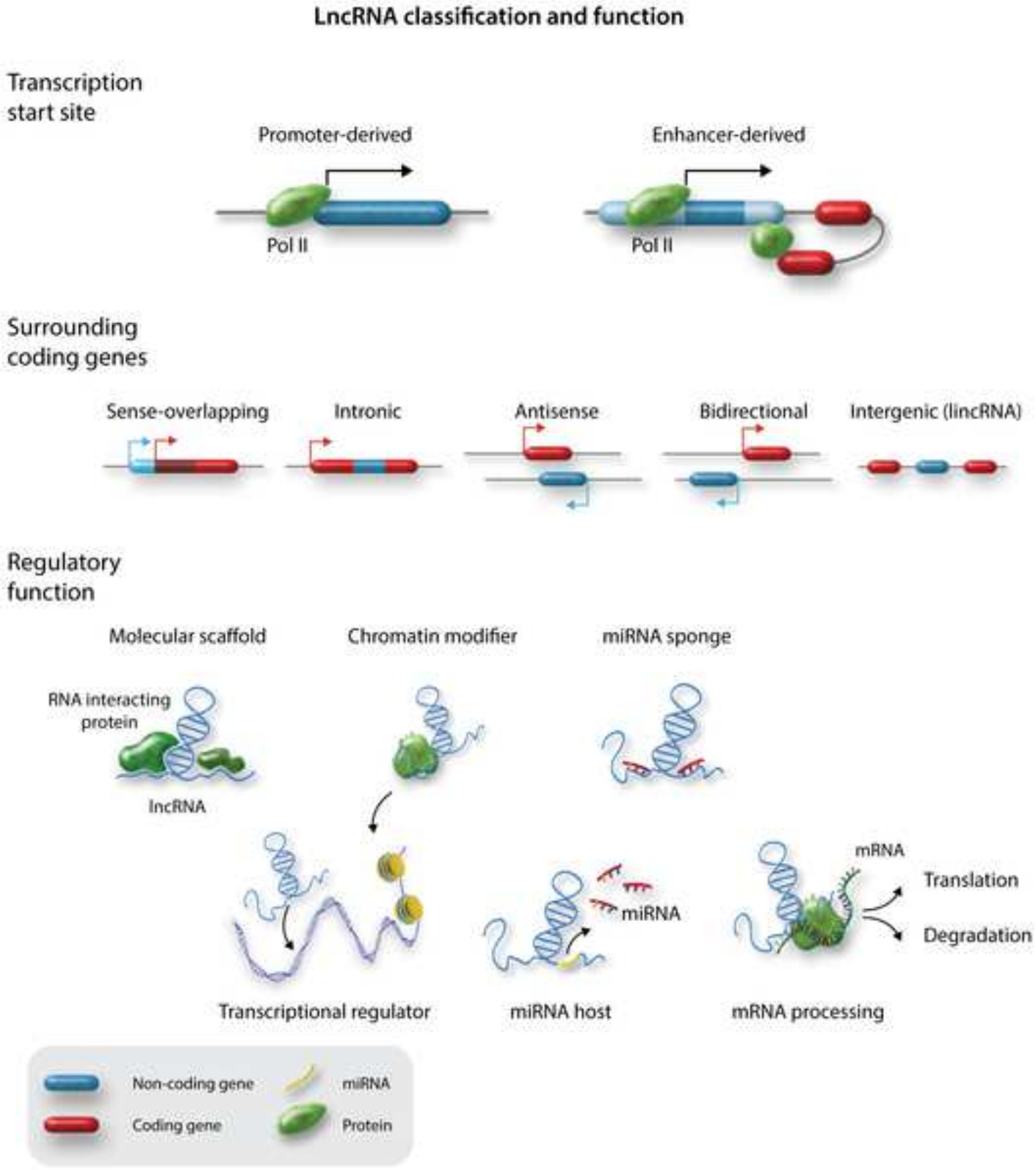
Figure 3 - Considerations for LncRNA Discovery. Common concepts to consider at the design stage for studies identifying lncRNA within sequencing data. These are generally tailored depending on the aims of the study, for instance it is often not necessary to profile for previously unannotated lncRNA across the whole transcriptome to find candidates of interest though studies with such scope can have greater power.

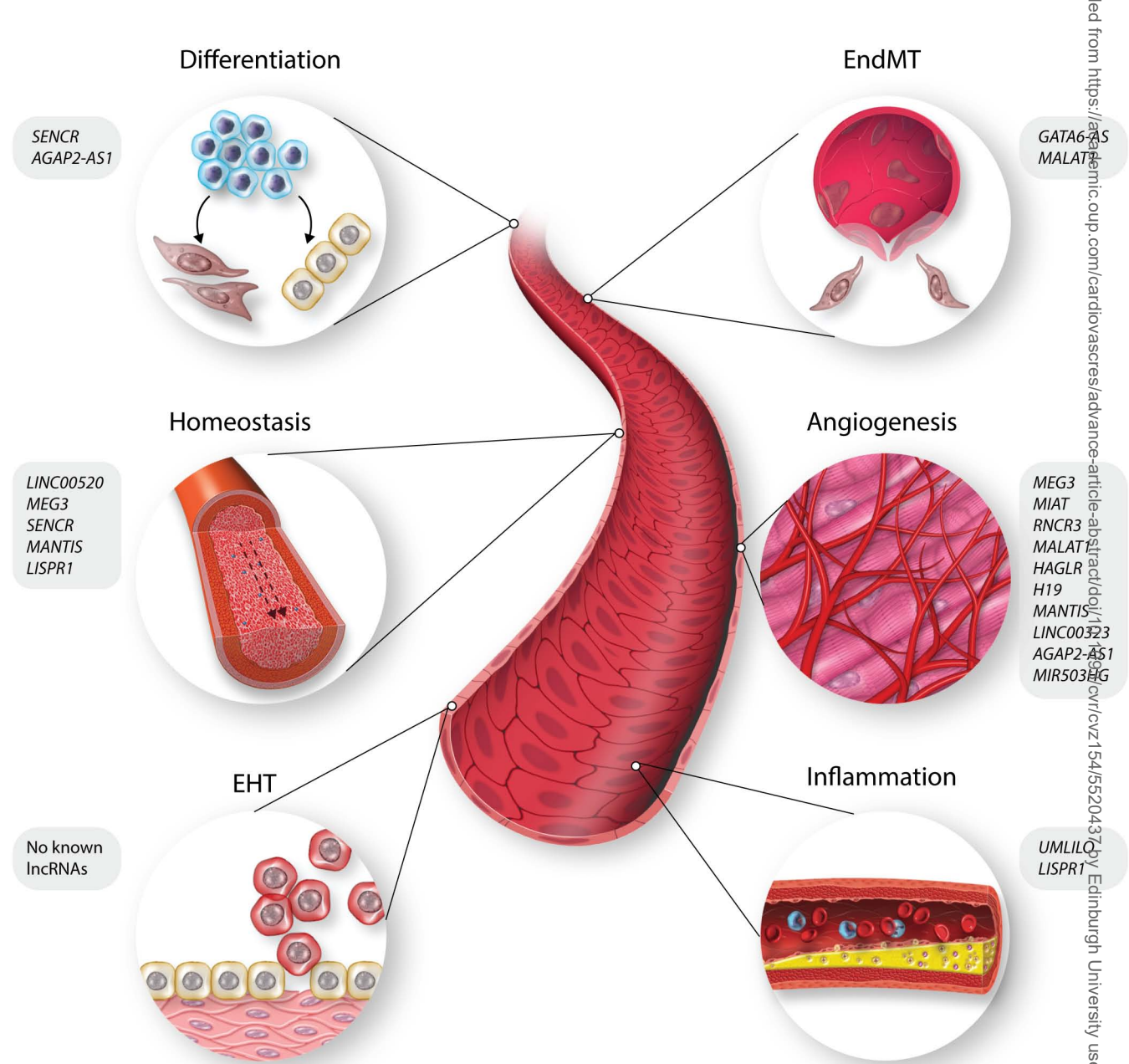
Figure 4 - RNA-focused Pulldown strategies. Workflow for the identification of lncRNA interactions using exogenous (left) or endogenous RNA pull-down strategies.

Table 1 - LncRNAs Associated with Endothelial Function

Gene Name	LncRNA Type	Sequence/ Synteny Conservation (GENCODE aligned to RLAR annotation)	Identification of LncRNA in Endothelial Context				Characterisation of LncRNA in Endothelial Context				
			Cell/ Tissue Type	Study Design	ID Tech.	Selection Strategy	Cell/ Tissue Type	Phenotypic Effect	Key Effectors + Interactions	Proposed Mechanism	References
AQAP3-AS1 (FUNSHCR)	antisense	Eutheria (Synteny: Ray-finned Fish)	Differentiated ECs	Differentiation from hESC to Vascular EC	RNAseq	Enriched in final stage of differentiation	Zebrafish/Mouse Embryo; HUVECs	Early vessel branching in zebrafish embryonic development, Maintenance of endothelial differentiation	TAL1, FOXC1	Unknown	[25]
GATA6-AS	antisense	Mammalia (Synteny: Ray-finned Fish)	HUVEC	Normoxia to Hypoxia (12 + 24 hours)	RNAseq	High abundance and upregulation relative to other lncRNA	HeLa/HUVEC	EndMT/Tip cell formation/Migration	LOXL2	Directs nuclear portion of LOXL2 to remove H3K4me3	[37]
HAGLR (STEEL, HOXD-AS1)	antisense	Ray-finned fish (Synteny: Ray-finned fish)	HUVEC/ HMVEC	Profiling of Primary Vessel Cell Types	LncRNA Custom Microarray	Enriched expression vs 4 Non EC types, proximity to Hox locus	HUVEC	Migration/Proliferation/ Apoptosis/Angiogenesis	KLF2, eNOS PARP1	Recruits epigenetic regulator PARP1 to target promoters	[38]
H19 (ASM)	lncRNA	Eutheria (Synteny: Eutheria)	HUVEC	Normoxia to Hypoxia (24 + 48 hours)	RNAseq	Hypoxia-sensitive mouse ortholog, sensitive to vascular injury	HAOEC	Supports hypoxia-induced Angiogenesis	Not examined here	Not examined here	[28]
LINC00520 (LEENE)	siRNA	No sequence ortholog (Synteny: Ray-finned fish)	HUVEC	Physiological (Pulsatile) vs Pathological (Oscillatory) shear stress	RNAseq	High upregulation relative to other lncRNA, correlation with eNOS	HUVEC	eNOS expression	Pol II, KLF4, Med1	Recruitment of Pol II to eNOS promoter	[51]
LINC00323 (C21orf130)	antisense	Catarrhini (Synteny: Eutheria)	HUVEC	Normoxia to Hypoxia (12 + 24 hours)	RNAseq + Non Coding Microarray	Strongest upregulation	HUVEC/HCAEC	Supports hypoxia-induced Angiogenesis	eIF4A3	Scaffold; indirect binding to GATA2	[27]
MALAT1 (HCN, LINC00047)	lncRNA	Vertebrates (Synteny: Ray-finned fish)	HUVEC	Profiling of HUVEC	RNAseq	Presence in other EC types	HUVEC	Supports hypoxia-induced Angiogenesis	S-phase cyclins, p21	Not examined in ECs	[26]
MANTIS (AK125871, ANXA4-AS)	antisense	Absent from GENCODE	HUVEC	siRNA targeting histone demethylase JARID1B	Exon-array	Upregulated after Histone Demethylase Depletion	HUVEC	Supports endothelial angiogenic function	BRG1, BAF155	Nucleosome remodelling via BRG1 interaction	[49]
MEG3 (GTL2, LINC00023)	lncRNA	Eutheria (Synteny: Ray-finned Fish)	HUVEC	Profiling of HUVEC during hypoxia + senescence	RNAseq/qRT-PCR	Upregulated in high HUVEC passage number	RF/6A (Primate retinal EC)	Supports Proliferation, Migration, Glucose-induced Apoptosis	Akt, EZH2/JARID2 (documented in ESCs)	Recruitment of EC-enriched histone demthylase JARID2	[26, 117, 118]
MIR503HG (H19X)	miR host	Eutheria (Synteny: Loke-finned fish)	HUVEC	Normoxia to Hypoxia (12 + 24 hours)	RNAseq + Non Coding Microarray	Strong upregulation	HUVEC/Ea.hy926	Supports proliferation and migration but no angiogenic effect	Not examined here	Host for miR-503	[27]
SENCR (FLJ1-AS1)	antisense	Eutheria (Synteny: Mammalia)	HUVEC/ hESC-EC	Hemogenic and directed differentiation of EC from hESC	qRT-PCR	Known enrichment in vascular cells	HUVEC/hESC-EC	Mesodermal/Endothelial Commitment, supports VEGF-induced angiogenesis + membrane integrity	CCL5, CXCL3L1, CKAP4, CDH5	Binds CKAP4, freeing CDH5 to stabilise adherens junctions	[34, 35, 53]
UMLILO (AC112918.3)	siRNA	Catarrhini (Synteny: Ray-finned fish)	HUVEC	TNFα treatment	Hi-C, ChIA-PET, ChIP-Seq	Interaction with chromosomal locus of CXCLs	HUVEC	Chemokine Expression	CXCL Locus	Facilitates MLL1-priming of CXCL locus via H3K4me3	[33]

Figure 1





Figure(s) 3